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(54) Title: HETEROLOGOUS BLOCK OLIGOMERS

(57) Abstract

Novel synthetic oligomers of unique three-dimensional structure comprising homologous and heterologous blocks, some of which may self-associate are described.

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HETEROLOGOUS BLOCK OLIGOMERS

This application is a continuation of United States application Serial No. 317,670 filed 1 March 1989, which in turn is a continuation-in-part of Serial No. 314,935 filed 24 February 1989.

FIELD OF INVENTION

This invention relates to synthetic oligomers of unique three-dimensional structure that combine the principles of polymer, peptide and synthetic DNA chemistry to provide rationally designed drugs, drug delivery systems, research tools and other products.

BACKGROUND OF THE INVENTION

Various heteropolymers are known (see, e.g., Seela, F. and Kaiser, K., Oligodeoxyribonucleotides containing 1,3-propanediol as a nucleoside substitute, Nucleic Acids Res. 15:3113-3129 (1987) and Connolly, B.A., The Synthesis of Oligonucleotides containing a primary amino group at the 5'-terminus, Nucleic Acids Res. 15:3131-3139 (1987)) and one example of a heterodimer exists. See Lemaitre, M., Bayard, B., and Leblue, B., Specific Antiviral Activity of a Poly(L-lysine)-Conjugated Oligodeoxyribonucleotide Sequence Complementary to Vesicular Stomatitis N Protein mRNA Initiation Site, Proc. Nat. Acad. Sci. U.S.A. 84:648-652 (1987). However, there is no known disclosure of molecules comprising linked heterologous blocks conforming to a predetermined or predictable three-dimensional structure.

Definitions

The following definitions apply to terms used in this specification and in the claims:

Block--An oligomer component of at least about 5 monomers, e.g., carbon atoms, amino acid residues or nucleotides which has an intrinsic tendency to self-associate inter se or with another block.

Unit--An oligomer component consisting of at least three blocks.

Oligomer--A molecule having from about 12 to about 150 monomers, e.g., carbon atoms, amino acid residues or nucleotides and comprising at least one unit.

Self-Association--The capability shared by two or more blocks to form a mutual linkage, e.g., the capability of homologous nucleic acid sequences to hybridize and of certain peptides to interact.

Homologous Block--One of a series of blocks whose members exhibit common properties, for example, one of a series of nucleic acid, peptide or organic polymer blocks. Organic polymer blocks may generally comprise straight or branched chain polyolefins such as polyethylene, polypropylene or polystyrene and other kinds of polymers which are not cross-linked.

Heterologous Block--One of a series of blocks whose members do not exhibit common properties.

Heterologous Block Oligomer (HBO)--An oligomer comprising at least one unit of the schematic formula A_1-B-A_2 in which the block B is heterologous with respect to at least one of the blocks A_1 and A_2 and is constrained into a generally looped configuration by the self-association energy of the blocks A_1 and A_2 or in which the blocks A_1 and A_2 are heterologous and are constrained into a spatially juxtaposed position by the internal self-association of the block B.

SUMMARY OF THE INVENTION

HBO's provide a broad spectrum of novel molecules. The new molecules may be predesigned to achieve objectives which have been realized, with some difficulty, if at all.

HBO detergents can be formed with self-associated DNA blocks joined by simple linker blocks. The properties of these detergent molecules can be exploited in several ways. Micelles formed primarily of the self-associated DNA blocks may permit the passage of any short, double-stranded DNA through the bloodstream. Antisense molecules and short duplex DNA's more intrinsically resistant to DNase could be delivered in this fashion. "Suicide" substrates of duplex DNAs can be constructed to target tissues and neoplastic or virus infected cells. The inclusion of a hydrophobic linker block in these HBOs facilitates diffusion or transport across cell membranes at the requisite site.

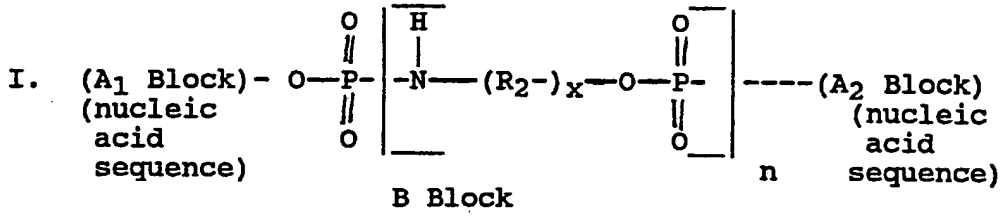
A variety of applications in protein purification, for example, hydrophobic interaction chromatography are apparent. More specifically the biospecificity of DNA sequences may combine with the capacity of the linker block to interact with such a chromatographic column.

DESCRIPTION OF THE FIGURES

Figure 1 is a two-dimensional generalized schematic depiction of an HBO in which the A_1 and A_2 blocks are self-associated.

For example, in such an HBO, the A_1 and A_2 blocks may be self-associating oligonucleotides, peptides or the like and the linker block B provides a preselected property, e.g., hydrophobicity. Alternatively, the linker block B may be nucleic acid sequences when the A_1 and A_2 blocks are self-associating peptides.

Formula I schematically represents one form of an HBO of the kind depicted by Figure 1:



in which R is an alkyl or aryl group of from about 1 to about 10 carbon atoms and x may be from about 3 to about 12. When x is greater than about 10, these HBO's are surfactive.

The number, n, of B block moieties depends upon the properties desired in the HBO. For many purposes n is from about 5 to about 20.

Figure 2 depicts an HBO in which the linker block B is internally self-associating, e.g., a homologous DNA sequence, flanked by A_1 and A_2 blocks such as peptides or organic polymers which do not self-associate and which are constrained in juxtaposition by the self-association energy of the linker block. Suitable DNA sequences for the B blocks of such HBOs include a stretch of hydrized nucleotides, generally a sequence of about 15 to 50 bases to provide the self-association energy appropriate to maintain the desired juxtaposition of the A_1 and A_2 blocks. Suitable peptides for use as A blocks contain from about 5 to about 30 residues. Suitable A block polymers include RNA, DNA, peptides or mixed RNA-DNA polymers having 12 to 150 nucleotides.

Appropriate selection of the A_1, B and A_2 blocks yields bioengineered catalysts in which catalysis is carried out by an appropriately constrained peptide, protein or RNA block. In addition, hydrophobic B blocks, e.g., amino-alkyl phosphonates, amino-aryl phosphonates, yield HBOs which are surfactants, particularly when the self-associating blocks are DNA.

Figure 3 is a copy of an autoradiograph illustrating the utility of an HBO as a human methyl transferase substrate.

Figure 4 illustrates a three nucleotide rule DNA methylation with DNA methyl transferase.

EXEMPLIFICATION OF THE INVENTION

EXAMPLE I

This example demonstrates the scope and significance of the invention. To do so, it identifies a specific question which has arisen in scientific research, describes the design of an HBO for use in answering the question, exemplifies the synthesis of the postulated HBO and shows that the synthesized HBO functions as intended.

The question addressed concerns the substrate specificity of the human DNA methyltransferase, i.e., whether the enzyme is capable of methylating across a gap in duplex DNA.

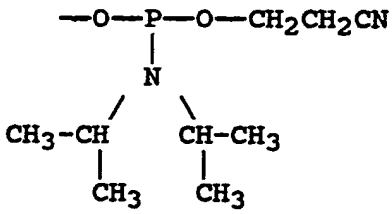
A 30 mer and a 13 mer were selected to provide a gapped duplex DNA as depicted by Formula II:

II. 5'GTCCACCAGATCC3' (13 mer)
3'CAGGTGGTCTAGGCCCGATGGACCGGAGCT5' (30 mer)

Prior experience indicated that the Formula I molecule might be unstable. Stability was achieved by linking the 13 mer and the 30 mer by a block effective to promote the annealing under the conditions contemplated for the reaction, i.e., 37°C, 50 m HEPES, pH7.1, 70 μM Spermine, 1.5 μM 5-adenosyl-L-(methyl-^{3}H) methionine [^{3}H AdoMet].

The design of the linker block raised two questions: (1) what moiety should be used to construct it, and (2) how long should it be? Hydrophobic moieties were rejected to preclude any test of the capability of the enzyme to interact with a detergent.

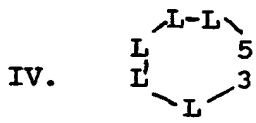
Cyanoethyl disopropyl phosphoramidite

III. 

was chosen as the linker moiety because it is appropriately protected for phosphoramidite synthesis and hence could be added to the termini DNA strands of the Formula I duplex.

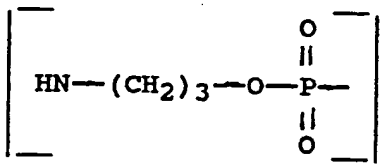
Computer modelling in which the desired molecule was constructed in BIOGRAPH was used to select the length of the linker group. Monomeric methane moieties were condensed to the 5′ phosphate oxygen of the 13 mer until a methylene chain $(-CH_2-)_n$ long enough to reach the 3′ hydroxyl of the 30 mer without producing a conformational change in the Formula I duplex DNA was constructed. The minimum length was determined to be 24($-CH_2-$)moieties. The selected linker monomer is somewhat longer than such moieties. Accordingly, a linker block consisting of

five linker units "L" was selected. The HBO to be synthesized is illustrated by Formula IV:



IV.

in which

L is 

Oligodeoxynucleotide Preparation and Characterization

Single strand oligodeoxynucleotides were synthesized using the phosphoramidite method (Sinha, N.D., et al., Nucleic Acids Res. 12:4539-4557 (1984)). The single stranded products were purified by polyacrylamide gel electrophoresis and high performance liquid chromatography as described by Tan, et al., Cold Spring Harbor Symp. on Quantitative Biology XLVII 383-391 (1982). The sequence of each of the oligodeoxynucleotides was verified using the method of Maxam and Gilbert.

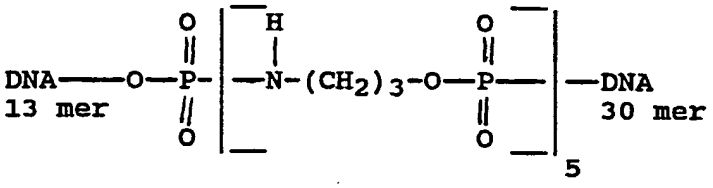
Duplex Oligodeoxynucleotides

Oligodeoxynucleotide concentrations were determined from absorbance at 260 nm. Duplex oligodeoxynucleotides were annealed from equimolar amounts of each single strand, as described in Smith, S.S., et al., Nucleic Acids Res. 15:6899-6916 (1987). The formation of duplex molecules was confirmed by electrophoretic separation of ^{32}P end-labelled duplexes (Smith, supra) on non-denaturing polyacrylamide gels. See Maniatis, T., et al. Biochemistry 14:3787-3794 (1975). End-labelled duplex molecules were further characterized by restriction analysis also as previously described in Smith.

The HBO was synthesized in a manner similar to the standard production of oligodeoxynucleotides. The amino group is protected by a monomethyoxyl trityl MMT or dimethoryl trityl DMT group and the phosphate group is simultaneously activated. After the MMT or DMT group was removed, the amino group was neutralized after each addition so that the next monomer could be added.

Formula V schematically depicts the HBO produced by this example:

V.



The HBO product of this example is an excellent substrate for human DNA methyltransferase as evidenced by the following test which is dependent on the spatial conformation of the molecule.

DNA(cytosine-5)methyltransferase was purified from human placentas as described in Smith, supra. When stored under the conditions described there, the enzyme loses less than 50% of its activity per year at -70°C. Two sets of assay conditions were employed. The unit of enzyme activity is defined in terms of the assay conditions used during enzyme purification with heat-denatured Micrococcus lysodeikticus DNA substrate (Smith supra). A unit of enzyme activity is the amount required to catalyze the incorporation of 1 pmole of methyl groups into TCA insoluble DNA in one hour at 37°C under those conditions.

For the determination of initial velocities with oligodeoxynucleotide substrates, the enzyme was dialyzed for 3 hours in a Hoefer microdialyzer (Health Products Inc., Rockford, Ill.) against 38 mM glycine, 17% v/v glycerol, 5 mM Tris, 10 mM β-mercaptoethanol, pH 7.8 at 4°C. The final reaction volume of 100 μl contained: 0.4 μg total DNA, 50 mM HEPES pH 7.0, 50 mM NaCl, 2 mM DTT, 75 μM Spermine, 10% v/v glycerol and 6.0 mM [^{3}H]AdoMet (Amersham, 15 Ci/mmole). Reaction mixtures were pre-incubated at 37°C for 15 minutes before the addition of 44 U of DNA methyltransferase to initiate the reaction. The reaction rate was linear under these conditions for 30 minutes. After 20 minutes of incubation, the reactions were stopped by the addition of 5 ml of cold TCA (5% w/v TCA containing 5 mM potassium pyrophosphate). Tritium incorporated into TCA insoluble DNA was determined as previously described (Smith, S.S., supra).

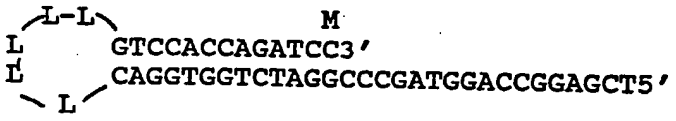
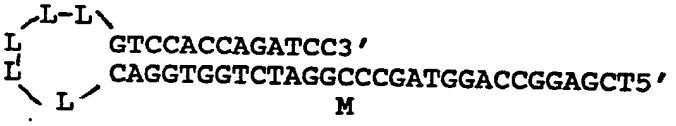
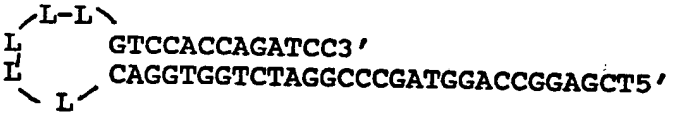
After enzymatic methylation of the substrate molecules with [^{3}H]5-adenosyl methionine as methyl donor, the labelled duplexes were cleaved with restriction endonucleases MboI and MspI. The products were electrophoretically separated and ^{3}H labelled DNA fragments were detected by fluorescence enhanced autoradiography as previously described in Smith, et al. The HBO molecule is refractory to digestion by MspI, consistent with the fact that the looped molecule cannot generate a complete duplex MspI site. On the other hand the same molecule is cleaved by MboI to about 70% completion. The cleavage product is only slightly shorter than the uncut molecule suggesting that MboI cleavage occurs on the cut site on the unmethylated portion of the

molecule to produce a molecule that is six nucleotides shorter (on the 3′ end) than the parent molecule. This partial cleavage pattern could be produced by the presence of the linkers in the loop, or it could mean that the fold-back structure does not form in a way that provides the enzyme with a completely recognizable cleavage site.

In any event, the DNA methyltransferase recognizes the structure and actively methylates it. The data given in Table I shows that the presence of a methyl group at the end of the short arm of the loop stimulates the reaction more than 100 fold (48L-1), while the presence of the methyl group at position 17 (bases numbered from the 5′ end of the molecule) (48L-2) does not stimulate the reaction rate.

These findings are consistent with a three nucleotide rule for DNA methylation which may be elucidated by reference to Figure 4. Referring to the figure, if enzymatic methylation occurs at cytosine (3), only the nucleotides shown in the L-shaped box are required. The enzyme must interact with cytosine (1) and its hydrogen bonded guanine (2) as it methylates cytosine (3). Guanine (4) is not required and can in fact be missing, alkylated, or replaced with any base. Even when the structure of a DNA substrate is not well understood, the application of this "L-Test for Methylation" is useful in predicting the methylation pattern applied in vitro. It clearly demonstrates that the DNA molecule that we have constructed is a well behaved enzyme substrate.

TABLE I

Molecule Code	Structure	DNA Methyl-transferase Substrate Activity (FMOLE/MIN)
48L-1		157
48L-2		11
48L-3		12

Enzymatic methylation of the HBO 48L-2 is demonstrated by the strong tritium signal in lanes 2 and 3 of the Figure 3 autoradiograph. To produce the autoradiograph HBO 48L-2 was exposed to human DNA methyltransferase in the presence of S adenosyl methionine as the methyl group donor. The reaction product was separated by polyacrylamide gel electrophoresis and the presence of enzymatically tritiated DNA was demonstrated by fluorescence enhanced autoradiography. Referring to Figure 3, lanes 1 and 7 include markers corresponding to 18, 24 and 30 bases. MboI and MspI identify restriction enzymes.

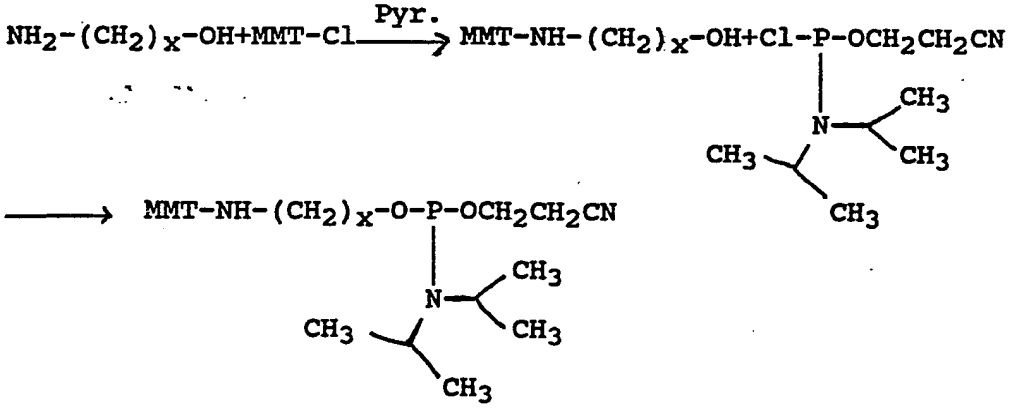
HBO's of all types may be synthesized by techniques known to the skilled man. Linker blocks B may be added to pre-self associated A_1 and A_2 blocks as typified by the example. Alternatively, all of the blocks may be separately synthesized and the desired HBO constructed from these prefabricated blocks. Such a procedure is preferred for the production of HBO's which involve the linkage of peptide and DNA blocks.

EXAMPLE II

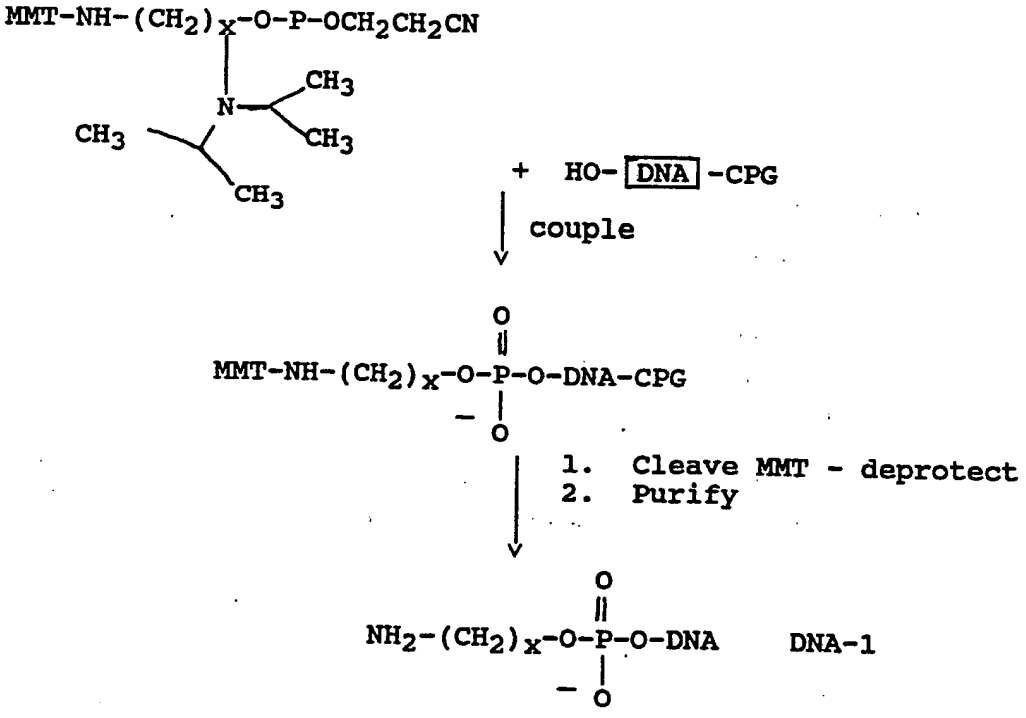
Example II illustrates one method for producing an HBO of schematic formula (DNA-2)-peptide-(DNA-1).

Synthesis of DNA-1

An amino alcohol is reacted with one equivalent of monomethoxytrityl chloride (MMT-Cl) in pyridine. The MMT-amino alkanol product is purified by chromatography and then reacted with an appropriate phosphitylating agent forming a cyanoethyl-diisopropylamino phosphite or a hydrogen phosphonate reagent or any other phosphite reagent known to the skilled worker. The product of this reaction is an activated phosphite reagent useful in any standard DNA synthesis machine. These reactions are illustrated by the following equation:

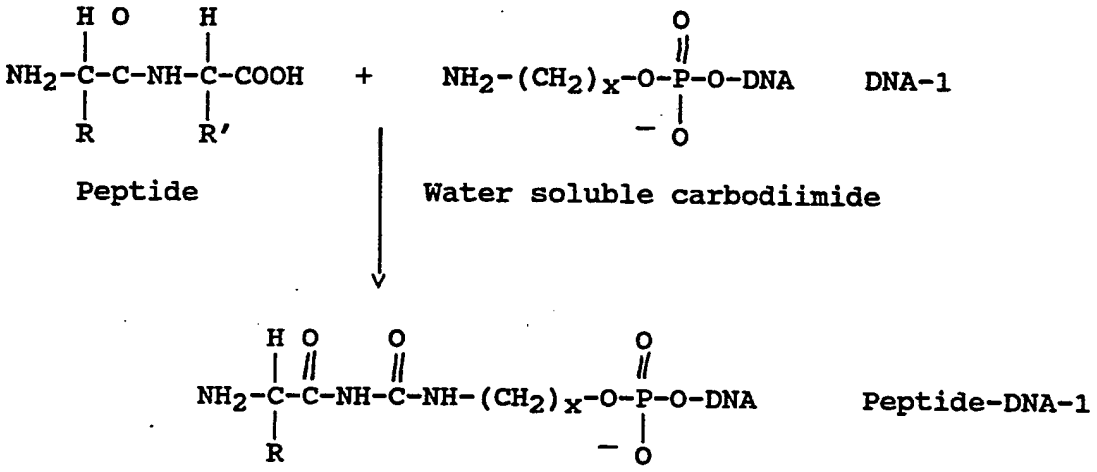


This activated phosphate reagent is c upled to the 5'OH of a growing DNA molecule synthesized in known manner on a solid support such as controlled pore glass (CPG). The MMT group is then removed with dichloroacetic acid or trichloroacetic acid and the amino group is neutralized to permit coupling to the next incoming phosphite reagent. Neutralization is accomplished by treating the growing DNA molecule with a dilute basic solution such as 1% triethyl amine in acetonitrile for a few seconds to convert the protonated amino group into a free amino group. These reactions are illustrated by the following equations:



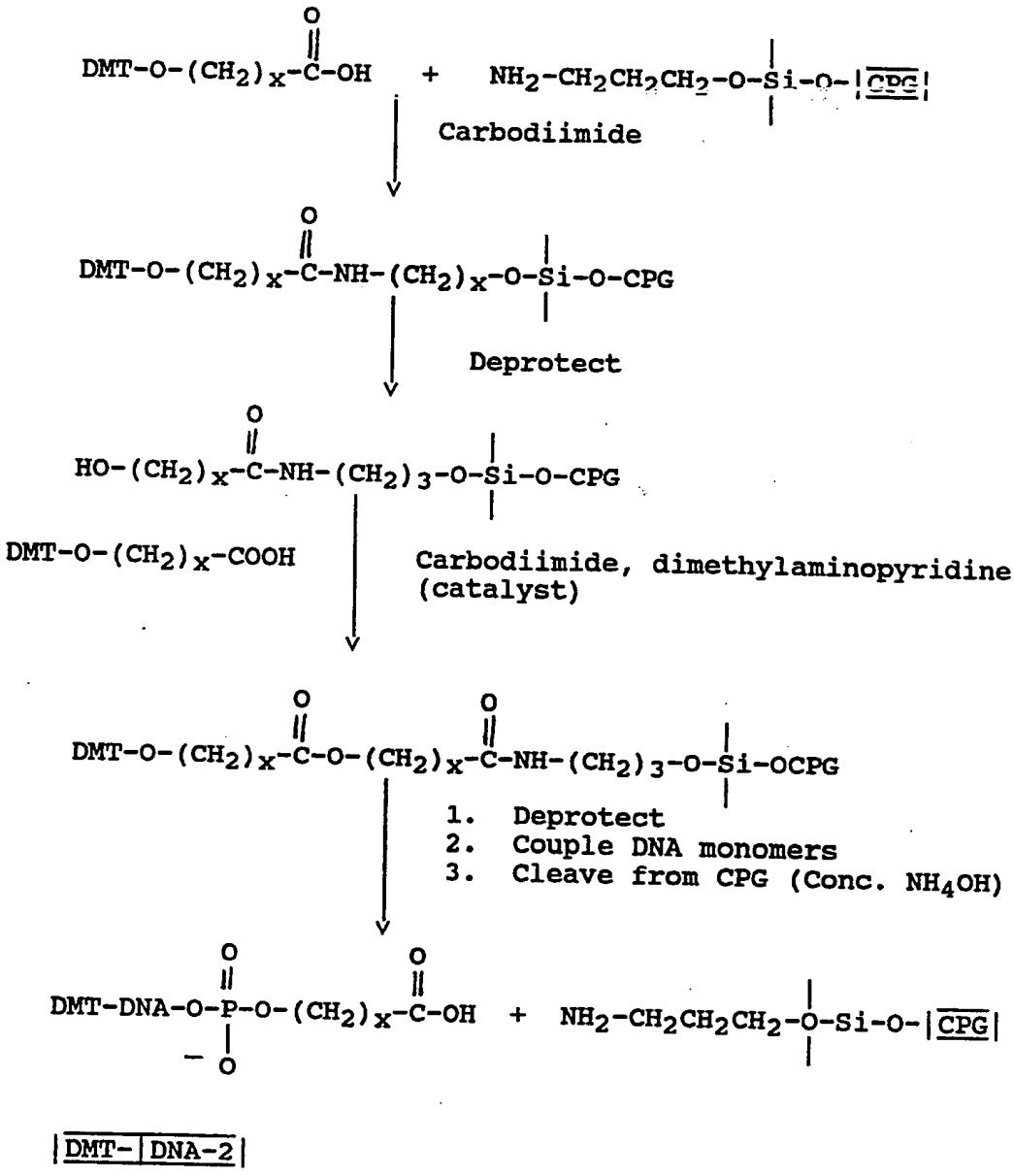
Synthesis of Peptide DNA-1

A peptide having a free amino and a free carboxyl group is coupled to DNA-1 in known manner, e.g., by use of a water soluble carbodiimide as illustrated by the following equation:



Synthesis of DNA-2

A DNA fragment with a free carboxylic acid on the 3′ end is synthesized on a solid support, for example, by connecting a DMT protected hydroxy carboxylic acid such as the DMT protected 6-hydroxyhexanoic acid to an amino-propyl CPG using a carbodiimide. After the coupling, the DMT group is removed in known manner using dichloroacetic acid in dichloromethane. A second reaction with a DMT protected hydroxy carboxylic acid is completed. The DMT group is again removed and again coupled with the DMT-protected 6-hydroxyhexanoic acid using a carbodiimide and dimethylaminopyridine to provide controlled pore glass as a support for the synthesis of DNA-2 in known manner. These reactions are illustrated by the following equations:


DMT-O-(CH2)x-C(=O)-OH + NH2-CH2CH2CH2-O-Si-O-|CPG|
Carbodiimide
DMT-O-(CH2)x-C(=O)-NH-(CH2)x-O-Si-O-CPG
Deprotect
HO-(CH2)x-C(=O)-NH-(CH2)3-O-Si-O-CPG
DMT-O-(CH2)x-COOH
Carbodiimide, dimethylaminopyridine (catalyst)
DMT-O-(CH2)x-C(=O)-O-(CH2)x-C(=O)-NH-(CH2)3-O-Si-OCPG
1. Deprotect
2. Couple DNA monomers
3. Cleave from CPG (Conc. NH4OH)
DMT-DNA-O-P(=O)(-O)-O-(CH2)x-C(=O)-OH + NH2-CH2CH2CH2-O-Si-O-|CPG|

|DMT-|DNA-2|

Synthesis of (DNA-2)-Peptide-(DNA-1)

After the synthesis is completed, the DNA-2 is deprotected in the standard way yielding a 5′ DMT-DNA-2. The 3′ carboxyl of this molecule is coupled to the amino group of the peptide-DNA-1 and the DMT group is removed to yield as the final product DNA-2-peptide-DNA-1.

5′ DMT-DNA-2 + Peptide-DNA-1

↓

DMT-DNA-2-peptide-DNA-1

↓ Deprotect DNA

DNA-2-peptide-DNA-1

WE CLAIM:

1. A heterologous block oligomer.

2. A heterologous block oligomer having a spatial configuration as generally depicted by Figure 1 or Figure 2.

3. An oligomer comprising at least one three block unit of schematic formula A_1-B-A_2 in which A_1 and A_2 are homologous self-associating blocks and B is a block heterologous with respect to blocks A_1 and A_2.

4. A heterologous block oligomer as defined by claim 3 in which blocks A_1 and A_2 are nucleic acid sequences or peptides.

5. A heterologous block oligomer as defined by claim 3 in which blocks A_1 and A_2 are nucleic acid sequences.

6. A heterologous block oligomer as defined by claim 5 in which at least one of the blocks A_1 and A_2 is DNA.

7. A heterologous block oligomer as defined by claim 5 in which one of the blocks A_1 and A_2 is DNA and the other is RNA.

8. A heterologous block oligomer as defined by claim 5 in which each of the A_1 and A_2 blocks are DNA blocks.

9. A heterologous block oligomer as defined by claim 8 in which the B blocks comprise an enzyme substrate.

10. A heterologous block oligomer as defined by claim 9 in which one of the A blocks comprises an antisense DNA.

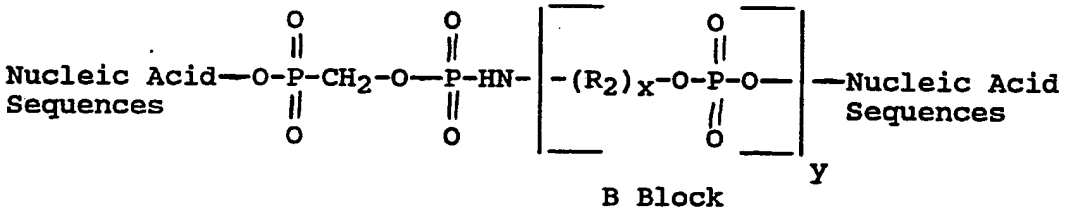
11. A heterologous block oligomer as defined by claim 5 in which the A_1 and A_2 blocks are DNA which comprise an antisense DNA.

12. A heterologous block oligomer as defined by claim 11 in which the B block comprises an enzyme substrate.

13. A heterologous block oligomer as defined by claim 3 in which block B is a peptide or a protein and the blocks A_1 and A_2 are nucleic acid sequences.

14. A heterologous block oligomer as defined by claim 3 in which block B is a biologically or enzymatically an active peptide, protein or RNA molecule and the blocks A_1 and A_2 are nucleic acid sequences.

15. A heterologous block oligomer as defined by claim 3 having the structural formula:



in which R is an alkyl or aryl group of from about 1 to about 10 carbon atoms and x may be from about 3 to about 12 and y is greater than 4.

16. A heterologous block oligomer as defined by claim 3 in which the B block is hydrophobic and the oligomer is surfactive.

17. A bioengineered delivery system comprising miscelles formed from concentrated heterologous block oligomers as defined by claim 16.

18. A heterologous block oligomer comprising at least one three block unit of schematic formula A_1-B-A_2 in which block B is internally self-associated and blocks A_1 or A_2 are maintained in juxtaposition by the internal self-association energy of block B.

19. A heterologous block oligomer as defined by claim 18 in which at least one of the A blocks is hydrophobic.

20. A heterologous block oligomer as defined by claim 19 in which the A blocks are peptides and the B block is a nucleic acid sequence.

21. A heterologous block oligomer comprising at least one three block unit of schematic formula A_1-B-A_2 and the block B is a peptide and the A_1 or A_2 blocks are self-associated organic moieties.

22. A heterologous block oligomer as defined by claim 21 in which the B block comprises a plurality of the repeat units of silk fibron or collagen.

23. A process for synthesizing a heterologous block oligomer having the schematic formula A_1-B-A_2 which comprises constructing a self-associated duplex of the A_1 and A_2 blocks and thereafter providing a linker B block in the form of a chain of repeating moieties which extends from a terminus of the A_1 block to the adjacent terminus of the A_2 block.

24. A process as defined by claim 23 in which said B block chain is preformed.

25. A process as defined by claim 24 in which said B block chain is produced by the addition of a first chain moiety to a terminus of the A_1 block and by thereafter sequentially adding B block chain moieties terminating with a final moiety bound to the adjacent terminus of the A_2 blocks.

26. A process for producing a heterologous block oligomer as defined by claim 21 in which the A_1, A_2 and B blocks are preformed and the A_1 and A_2 blocks are thereafter attached to the termini of the B block.

27. A process which comprises delivering a therapeutic agent to a target in the body of a mammal which comprises incorporating said therapeutic agent in a delivery system as defined by claim 17 and injecting said system containing said therapeutic agent into the bloodstream of said mammal.

FIG. 1

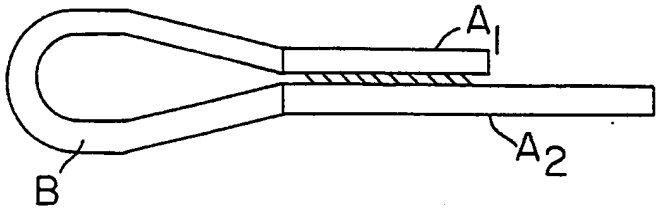


FIG. 2

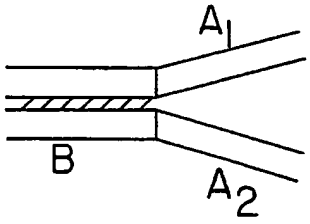
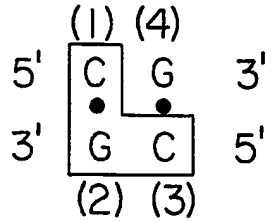


FIG. 4



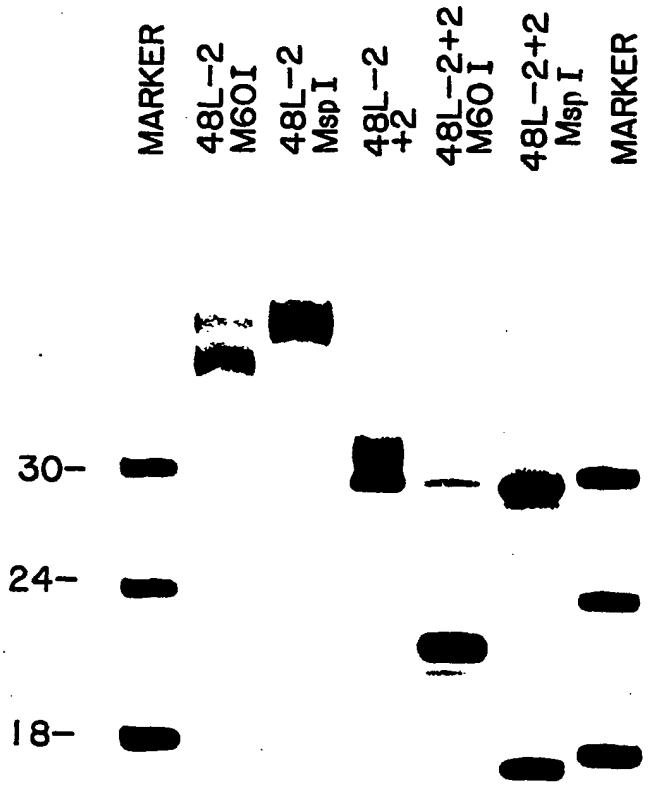

MARKER
48L-2 M60I
48L-2 Msp I
48L-2 +2
48L-2+2 M60 I
48L-2+2 Msp I
MARKER
30-
24-
18-

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00884

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5) : A61K 31/100; C07H 15/00
U.S. Cl : 536/27,28,29

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	536/27,28,29

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	US,A 4,415,732 (CARUTHERS ET AL) 15 Novmeber 1983, See Entire Document.	1-16,18-26
Y	US,A 4,757,141 (FUNG ET AL) 12 July 1988 See Column 2.	1-26
Y,E P	US,A 4,904,582 (TULLIS) 27 February 1990 See Entire Document.	27

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 May 1990	06 JUL 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	James O. Wilson